Master's Thesis

The novel E3 ligase of PPARγ TRIM25 regulates adipocyte differentiation

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12/07/2017

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Abstract

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-dependent transcription factor which regulates glucose homeostasis and adipocyte differentiation. Its transcriptional activity is regulated by not only ligands but also post-translational modifications (PTMs). In this study, we demonstrate a novel E3 ligase of PPARγ, TRIM25 directly induces ubiquitination of PPARγ followed by proteasome-dependent degradation. During the adipocyte differentiation, both mRNA and protein expression of TRIM25 significantly decreased and negatively correlated with the expression of PPARγ. Stable expression of TRIM25 reduces PPARγ protein levels, but not mRNA expression, and suppressed adipocyte differentiation in 3T3-L1 cells. In contrast, specific knock-down of TRIM25 increases PPARγ protein levels and stimulates adipocyte differentiation. Furthermore, TRIM25 knock-out mouse embryonic fibroblast (MEFs) shows an increased ability for adipocyte differentiation compared with wild-type MEFs. Taken together, these data indicate that TRIM25 is a novel E3 ubiquitin ligase of PPARγ, and depict TRIM25 as a novel target for PPARγ-involved metabolic diseases.

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Abbreviations

BMI : Body mass index

WHO : World Health organization

C/EBPs : CCAAT/enhancer binding proteins

PPARγ : peroxisome proliferator-activated receptor γ

TZDs : thiazolidinediones

PON1 : paraoxonase 1

PTMs : post-translational modifications

MAVS : Mitochondrial antiviral-signaling protein

MEFs : mouse embryonic fibroblast

aP2 : fatty acid-binding protein

Fasn : fatty acid synthase

LPL : lipoprotein lipase

RXR : retinoid X receptor

DR1 elements : DNA repeats of the sequence AGGTCA

NCOR1 : nuclear receptor corepressor 1

SMRT/NCOR2 : silencing mediator for retinoid and thyroid receptor

SRCs : steroid receptor coactivator

PGC1s : PPARγ coactivator 1s

HATs : histone acyltransferases

MAPK : mitogen-activated protein kinase

CDK5 : cyclin-dependent kinase 5

A/B : Transcriptional regulation region of PPARγ

DBD/H : DNA binding domain

LBD : Ligand binding domain

DIO : diet-induced obesity model

TRIM : Tripartite motif

RIG-1 : retinoic acid inducible gene 1

E1 : ubiquitin activating enzyme

E2 : ubiquitin-conjugating enzyme

E3 : ubiquitin protein ligase

LC-MS/MS : liquid chromatography combined with tandem mass spectrometry

CHX : cycloheximide

TG : triglyceride

GLUT4 : glucose transporter-4

Ⅰ. Background

1-1. Obesity

Obesity is defined excessive accumulation of fat in body. Numerically, Body mass index (BMI) which is an index of weight for height is commonly used to classify obesity and obesity is a BMI greater than or equal to 30 (Fig.1-1). [1] Recently, Obesity has increased as people's living conditions have changed. According to World Health organization (WHO), The fundamental cause of obesity is an energy imbalance between calories consumed and calories expended. Obesity is preventable because it is not communicable disease by reducing the fat, sugar and salt content food and engage in regular physical activity. However, it is hard to maintain lifestyle because people who take more energy dense foods and have physical inactivity due to many forms of work have been increasing in worldwide.[1-5] In addition to increased future risks, obese people experience breathing difficulties, increased risk of fractures, hypertension, early markers of cardiovascular disease, insulin resistance and psychological effects.[5]

1-2. metabolic syndrome

Metabolic syndrome is associated with abdominal obesity, blood lipid disorders, inflammation, insulin resistance or full-blown diabetes, and increased risk of developing cardiovascular disease.[6] WHO analyzed that Obesity is complex disease and is linked to more deaths compared with normal because it is related metabolic syndrome such as diabetes, ischemic heart disease and certain cancer (Fig.1-2).[7] Many studies suggested that obesity might be causative factors in metabolic diseases and cancers.

1-3. Adipogenesis

Adipogenesis is one of the process of cell differentiation, which means that preadipocyte become adipocyte. Adipocyte differentiation is a complex process involving dramatic changes in cell morphology and gene expression. Many transcription factors have been identified as potential regulators of this process.[8] CCAAT/enhancer binding proteins (C/EBPs) are the master regulators of adipogenesis.[9] C/EBP-β and C/EBP-δ are expressed at the early stage of adipogenesis [10] and in turn they directly induce expression of peroxisome proliferator-activated receptor γ (PPARγ) and C/EBP-α, the key transcriptional regulators of adipogenesis which induce their own expression and activate a number of target genes (Fig.1-3).[11] Although studies continue to increase our understanding of adipogenesis, as yet, there is little information about what controls the death or turnover of adipocytes *in vivo*.[12]

1-4. PPARγ

Peroxisome proliferator-activated receptor γ (PPARγ) mainly expressed in adipose tissues and it regulates fatty acid storage, glucose metabolism and adipogenesis. PPARγ has been clinically related in numerous diseases of including obesity, diabetes, atherosclerosis, and cancer. It also known as glitazone receptor because glitazone is ligand of PPARγ such as thiazolidinediones (TZDs) which are known class of anti-diabetic drugs. PPARγ agonists have been used in the treatment of hyperlipidemia and hyperglycemia,[13] and it had reported that PPARγ decreases the inflammatory response of many cardiovascular cells, particularly endothelial cells.[14] PPARγ increase synthesis and release of paraoxonase 1(PON1) from the liver, reducing atherosclerosis (Fig 1-4).[15] However, TZDs have unwanted side effects, such as weight gain, heart failure.[16] Besides of ligands, post-translational modifications (PTMs), including phosphorylation, SUMOylating, acetylation, and ubiquitination of PPARγ are considered as one of the major processes regulating the transcriptional activity of PPARγ.[17] Many researchers expected it probably involve changing the way focusing on downstream effectors of PPARγ-mediated insulin sensitization, targeting specific post-translational modifications of PPARγ and selectively agonizing or antagonizing PPARγ in specific tissues.

1-5. TRIM25

TRIM25 is known about E3 ubiquitin ligase. Polyubiquitination is the formation of a ubiquitin chain on a single lysine residue on the substrate protein.[18] These chains are made by linking the glycine residue of a ubiquitin molecule to a lysine of ubiquitin bound to a substrate. Lysine 48-linked polyubiquitin chains target proteins for destruction, by a process known as proteolysis. they allow the coordination of cellular signaling such as endocytic trafficking, inflammation, translation, and DNA repair.[19] It was reported that TRIM25 induces ubiquitination of RIG-1, which is a cellular sensor of RNA virus infection and crucial for the cytosolic RIG-I signaling pathway to elicit host antiviral innate immunity. TRIM25 interacts with the N-terminal CARDs of RIG-I, this interaction effectively delivers the Lys 63-linked ubiquitin moiety to the N-terminal CARDs of RIG-I, resulting in a marked increase in RIG-I downstream signaling activity.[20] TRIM25 also induces the Lys 48-linked ubiquitination of Mitochondrial antiviral-signaling protein (MAVS) and degradation by the proteasome is involved in type I interferon production after activation of the antiviral RIG-I-like receptors.[21] 14-3-30 is responsible for reduced cell growth, as a factor that interacts with TIM25, it has been revealed that TRIM25 is an E3 ubiquitin ligase that targets the proteolysis of 14-3-30 .[22]

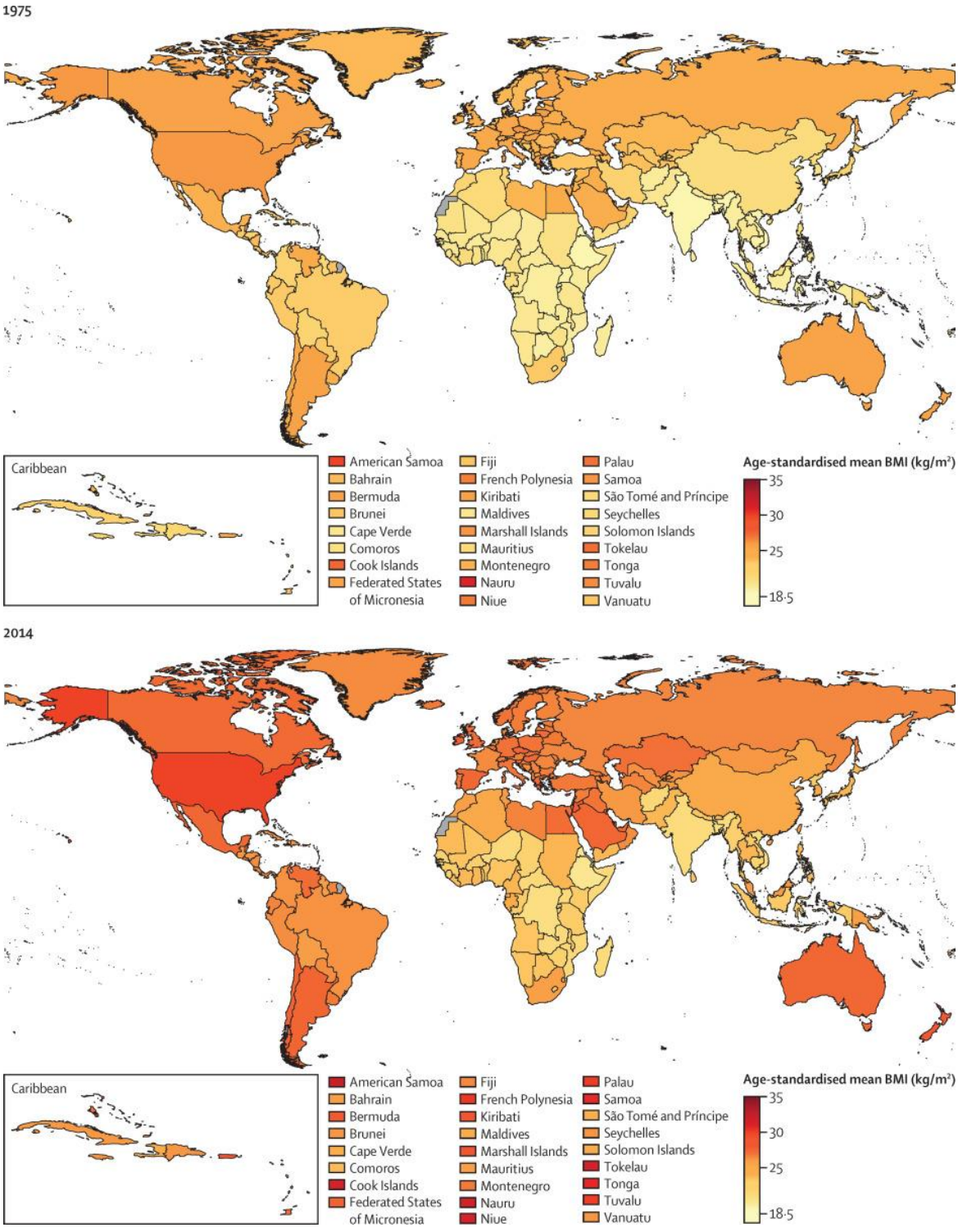

Figure 1-1. Age-standardized mean BMI in men by country in 1975 and 2014

Circulation **2013,** *128* (15), 1689-1712.

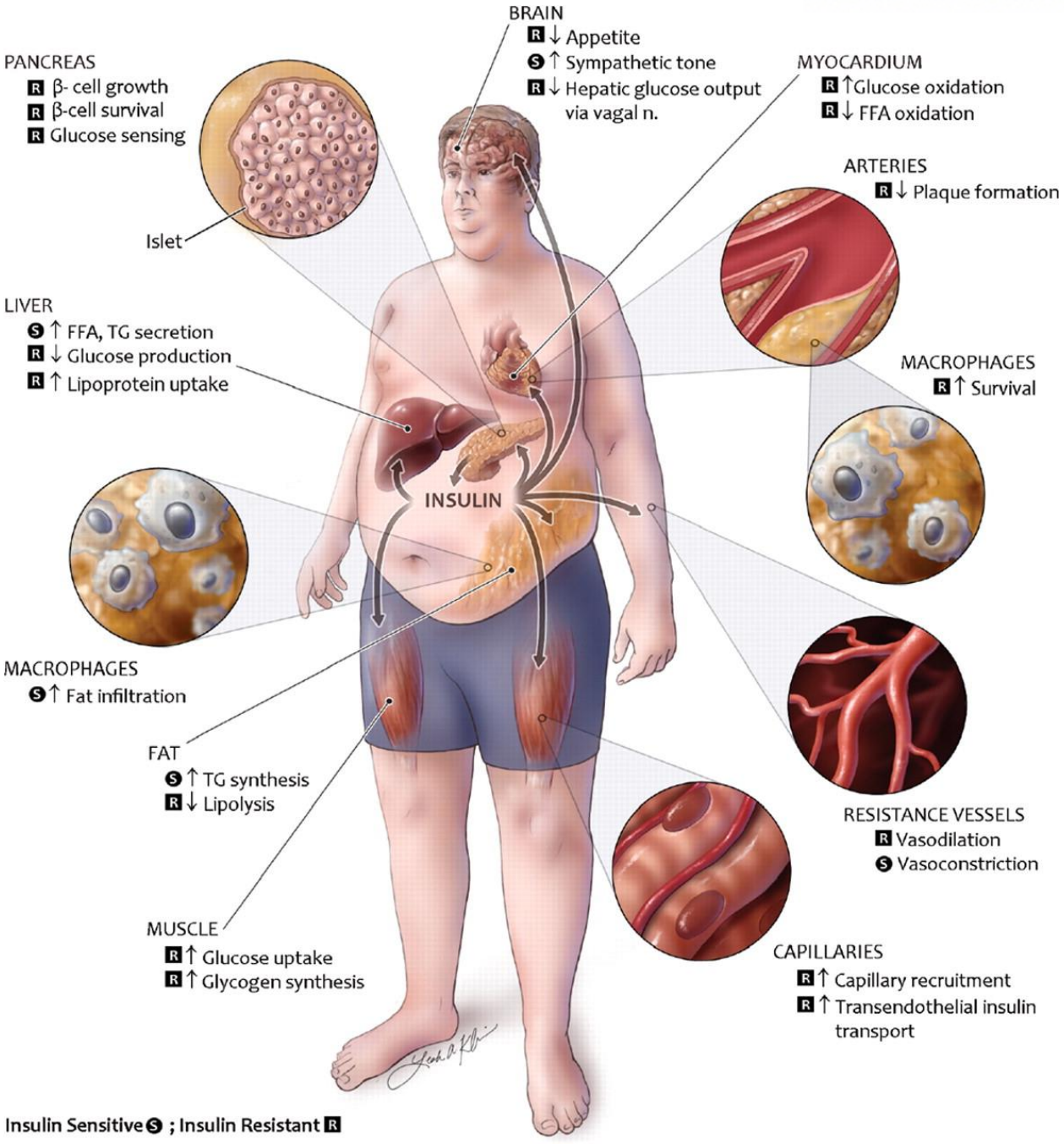

Figure 1-2. Physiology of insulin signaling in metabolic syndrome

Geneva: World Health Organization **2009,** *15*.

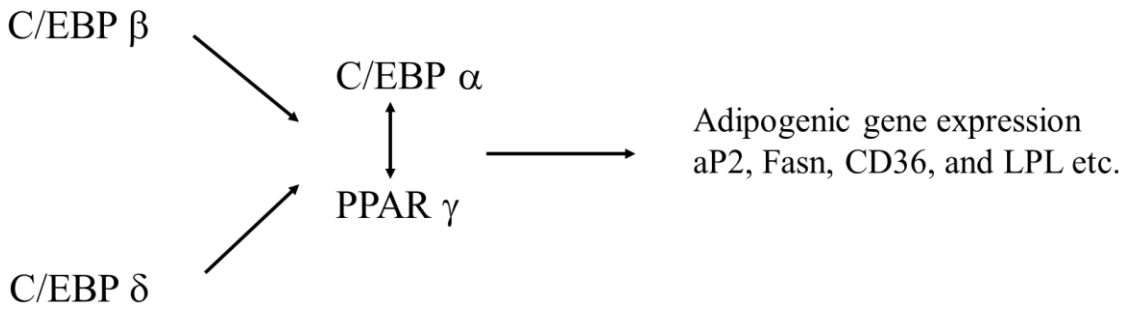

Figure 1-3. Roles of PPARγ and C/EBPα in adipogenesis

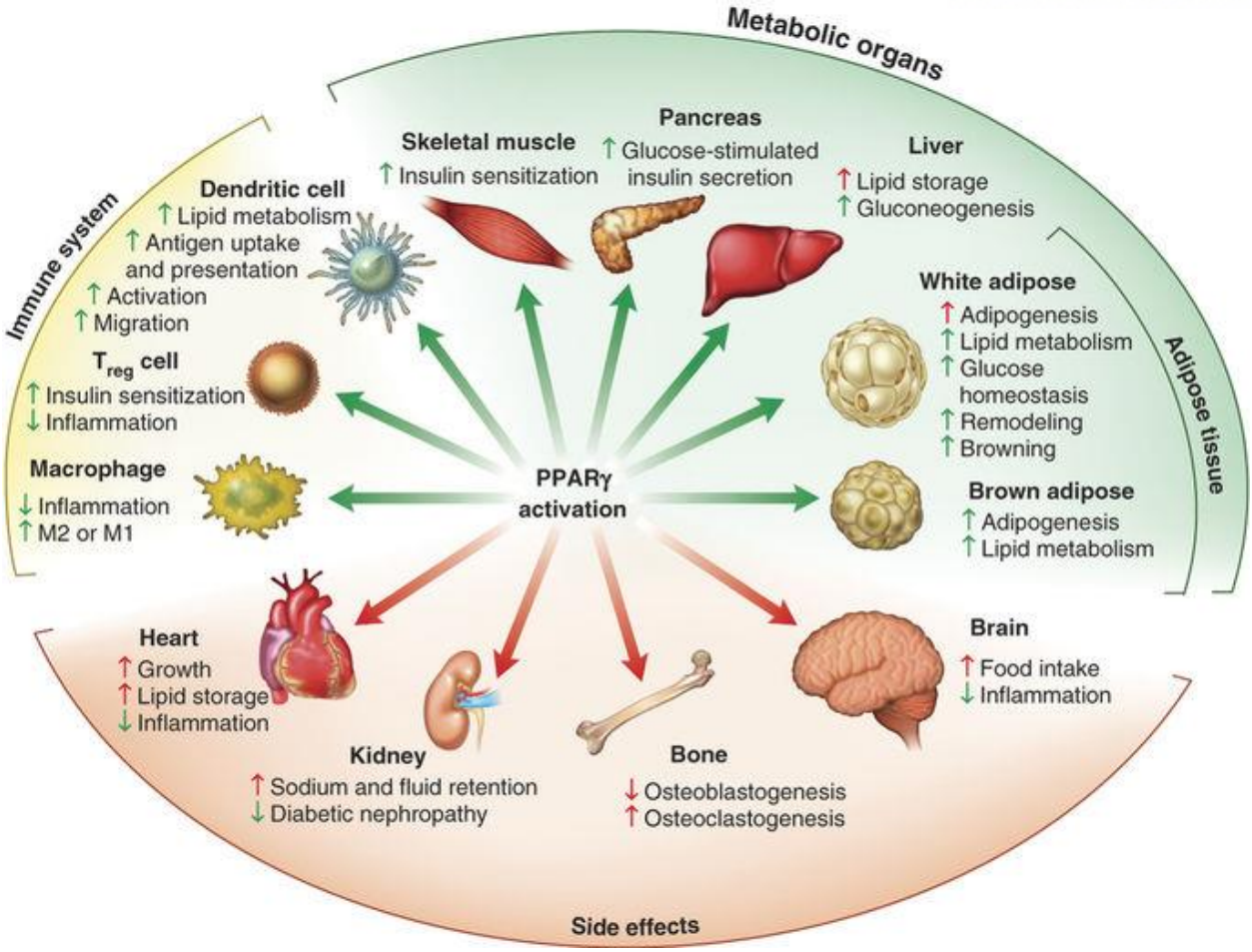

Figure 1-4. Known effects of PPARγ activation

Nature Medicine **99**, 557–566 (2013).

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Ⅱ. TRIM25, a novel E3 ligase of PPARγ prevents adipocyte differentiation

2-1. Introduction

Adipose tissue has a pivotal role in storing excess energy and is a center for energy metabolism.[23] Excess body fat is considered one of the major causes of insulin resistance, dyslipidemia, type 2 diabetes, certain types of cancer, and cardiovascular disease.[24] In obesity, adipocytes exhibit the alteration of the status of energy homeostasis not only to store the energy but also to generate and secrete hormones and cytokines called adipokines.[25] For instance, the expression of insulin-resistance-inducing adipokines including TNF-α, IL-1, or resistin were increased in adipose tissue from obese individuals, but the production of the insulin-sensitizing hormone, adiponectin or adipsin was decreased.[26] Furthermore, the defects in adipocyte differentiation or functions increased the probability of metabolic disorders. Thus, understanding of detail molecular mechanism in adipose tissue biology, especially adipocyte differentiation may provide the insight for treating obesity and metabolic syndrome.

Adipocyte differentiation is tightly controlled by a series of transcriptional factors. A number of studies have demonstrated that peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) are the master regulators of adipogenesis.[9] The first stage of adipogenesis consists of the induction of C/EBP-β and C/EBP-δ,[10] and in turn they directly induce expression of PPARγ and C/EBP-α, the key transcriptional regulators of adipogenesis.[11] The importance of C/EBP-β and C/EBP-δ during adipogenesis is demonstrated by loss-of-function and gain-of function genetic studies in mice.[27] Mice lacking either C/EBP-β or C/EBP-δ exhibit a reduced mass of white adipose tissue.[28] In addition, it has been well studied that PPARγ and C/EBP-α induce their own expression and activate a number of target genes such as fatty acid-binding protein (aP2), fatty acid synthase (Fasn), CD36, and lipoprotein lipase (LPL).[29] C/EBP-α-null embryonic fibroblast cells fail to undergo adipogenesis, but this defect can be restored by overexpression of PPARγ.[30] Conversely, forced expression of C/EBP-α in PPARγ-null embryonic fibroblast cells does not allow the cells to differentiate.[31] These experiments demonstrate that PPARγ is the key transcriptional regulator of adipogenesis, and it is both sufficient and necessary for fat cell differentiation.

It has been well known that the transcriptional activity of PPARγ is up-regulated by its ligands such as thiazolidinediones (TZDs).[32] PPARγ specifically makes a heterodimer with retinoid X receptor (RXR) to DNA repeats of the sequence AGGTCA (DR1 elements), and PPARγ/RXR heterodimer can regulate variety of target genes in different cells.[33] In the resting state (without

PPARγ ligands), PPARγ preferentially binds to nuclear receptor corepressor 1 (NCOR1) and silencing mediator for retinoid and thyroid receptor (SMRT/NCOR2).[34] These complexes recruit chromatin-modifying enzymes such as histone deacetylases to repress transcription.[35] However, when the ligands activate PPARγ, corepressors are dissociated with PPARγ and coactivators such as steroid receptor coactivator (SRCs), PPARγ coactivator 1s (PGC1s), histone acyltransferases (HATs), and the Mediator complex are recruited and interact with PPARγ and promote gene transcription.[36] Besides of ligands, post-translational modifications (PTMs), including phosphorylation, SUMOylating, acetylation, and ubiquitination of PPARγ are considered as one of the major processes regulating the transcriptional activity of PPARγ.[17] Phosphorylation at Ser112 of PPARγ is modified by mitogen-activated protein kinase (MAPK) and suppresses PPARγ transcriptional activity and adipocyte differentiation.[37] In contrast to Ser112, PPARγ phosphorylation at Ser273 by cyclin-dependent kinase (CDK5)/ERK did not change its transcriptional activity, but its phosphorylation has important implications for the treatment of type 2 diabetes.[38] SUMOylating at Lys107 of PPARγ blocks transcriptional activity and Lys395 of PPARγ represses inflammatory genes expression by blocking NF-κB activation.[39] PPARγ is also ubiquitinated and degraded in a proteasome-dependent manner.[40] These studies have shown that post-translational modifications of PPARγ are important factor for physiological role of PPARγ. Thus, the characterization of the novel PTMs of PPARγ will provide important insights for understanding the physiological functions of PPARγ in both adipogenesis.

Tripartite motif (TRIM) proteins are defined as E3 ubiquitin ligases as they contain a ring-finger domain.[41] So far, there are more than 77 known TRIM proteins in human.[42] They are involved in a broad range of biological processes and the dysfunction of TRIM proteins results in variety of pathophysiological conditions.[43] It has been known that TRIM25 (also known as EFP) is one of the downstream targets of estrogen receptor α. The expression of TRIM25 is upregulated in response to estrogen, and it is thought to mediate estrogen actions in breast cancer as a primary response gene.[44] In addition, TRIM25 induces the ubiquitination of retinoic acid inducible gene 1 (RIG-I) and regulates host anti-viral innate immunity.[45]

In this study, we report the novel roles of TRIM25 in regulating metabolic pathways by mediating PPARγ ubiquitination and proteasome-dependent degradation. The physiological function of TRIM25 on PPARγ protein stability is further confirmed in both 3T3-L1 adipocytes and TRIM25 knock-out MEFs, suggesting that TRIM25 directly regulates PPARγ protein stability, which controls adipocyte differentiation. Together, these results demonstrate that TRIM25 may be a potential therapeutic target in PPARγ-mediated metabolic diseases such as obesity and type 2 diabetes.

2-2. Materials and Methods

Cell culture

3T3-L1 and HEK-293 cells were obtained from ATCC (VA, USA) and cultured in in Dulbecco's modified Eagle's medium (DMEM) containing Bovine Serum (Gibco BRL, NY, USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C under a humidified 5% CO2 atmosphere. PPARγ-deficient MEFs have been described previously.[11] Adipocyte differentiation was induced by treating cells with DMEM medium containing 10% fetal bovine serum (FBS; Gemini Bio-Products, Inc.), 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone and 850 nM insulin. Two days after the induction, cells were switched to the maintenance DMEM medium containing 10% FBS and 850 nM insulin. Lipid accumulation in the cells was detected by Oil Red O (Sigma) staining. Mouse embryonic fibroblast cells (MEFs) and TRIM25 deletion MEFs were kindly provided by Dr. Kyung-soo Inn.[46]

Plasmid constructs and shRNAs

The mammalian expression vector for Flag-epitope tagged wild-type PPARγ, Which was described previously.[11] Murine TRIM25 and its deletion mutants were kindly provided by Dr. V. Narry Kim.[47] The sequence used for lentiviral shRNA expression vector (pLKO.1; GE Healthcare, Dharmacon) targeting TRIM25 is 5' - AAACCCAGGGCTGCCTTGGAAAAG - 3'. For lentivirus production, 3T3-L1 cells (ATCC, VA, USA) were transfected with 10 μg lentiviral vectors. Following infection of the cells with the lentivirus, cells were selected by incubation with 1 μg/ml puromycin.

Binding assay and immunoprecipitation

Immobilized GST-fused proteins (PPARγ-AB, DBD/H, LBD, WT, TRIM25-Truncated mutant) with glutathione–agarose was incubated with PPARγ or TRIM25-expressing cell lysates for 2 h at 4 °C. Protein complexes were pulled-down by centrifugation and washed four times with binding buffer. Precipitates were detected by immunoblotting using anti-PPARγ or TRIM25 antibodies. For analyzing interaction between PPARγ and TRIM25, HEK-293 cells expressing PPARγ, TRIM25 or their mutants were lysed with binding buffer and total cell lysates were incubated with FLAG M2 agarose (Sigma, MO, USA) at 4 °C. Immunoprecipitants or total cell lysates were analyzed with specific antibodies as indicated.

Reporter gene assay

HEK-293 cells were transfected with pDR-1 luciferase reporter plasmid, PPARγ, RXRα and

pRL-Renillia using Lipofectamine 2000 (Invitrogen, CA, USA). Following an overnight transfection, the cells were treated with rosiglitazone for 24 h. The cells were harvested, and reporter gene assays were carried out using the Dual-Luciferase kit (Promega, WI, USA). Luciferase activity was normalized to Renillia activity.

Gene expression analysis

Total RNA was isolated from cells or tissues using TRIzol reagent (Invitrogen, CA, USA). RNA was reverse-transcribed using an ABI Reverse Transcription Kit. Quantitative PCR reactions were performed with SYBR green fluorescent dye on an ABI9300 PCR machine. Relative mRNA expression was determined by the ΔΔ-Ct method normalized to 60S acidic ribosomal protein P0 (RPLP0) levels.

Identification of the PPARγ binding complexes

PPARγ or an empty vector were grown to confluence and were induced differentiation as described above. At day 6 of differentiation, cells were homogenized in a hypotonic solution (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, protein inhibitors) and spun at 1000 g for 15 min to pellet the nuclei. The nucleus fractions were resuspended with a hypertonic solution (20 mM HEPES at pH 7.9, 400 mM NaCl, 1.5 mMMgCl2, 0.5 mM DTT, 0.2 mM EDTA, 20% glycerol, protein inhibitors) for 30 min to extract nuclear proteins. Samples were spun at16, 000 g for 30 min and dialyzed against a binding buffer containing 20 mM HEPES (pH 7.9), 150 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM DTT, and 0.1 mM PMSF. The nuclear extracts were incubated overnight with FlagM2 agarose (Sigma, MO, USA), washed in a binding buffer (250 mM KCl), and then eluted by incubating with 3X Flag peptide (0.2 mg/ml). The eluted materials were separated in a 4–12% acrylamide gradient gel, and visualized by silver staining. Gel-resolved proteins were excised, digested with trypsin, and individually analyzed in an Orbitrap ELITE mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). We separated peptides in a C18 reverse phase HPLC column (150 mm × 75 μm i.d.) using an acetonitrile/0.1% formic acid gradient of 66 min at a flowrate of 300 nl/min. For MS/MS analysis the precursor ion scan MS spectra (from m/z 400 to 2000) were acquired in the Orbitrap with resolution R = 60,000 at m/z = 400 with an internal lock mass. The 20 most intense ions were isolated and fragmented in the linear ion trap by collisionally induced dissociation (CID). All MS/MS spectra were searched against the Uniprot protein sequence database by SequestHT.

In vitro ubiquitination assay

FLAG-TRIM25 was transiently expressed in HEK-293 cells, purified by immunoprecipitation using anti-FLAG M2 affinity gel (Sigma-Aldrich, A2220), and eluted by adding FLAG peptide (Sigma-Aldrich, F3290) according to the manufacturer's instructions. Bacterially expressed recombinant GST-PPAR2 was incubated with 200 ng E1 (UBE1, E-305, Boston Biochem, Cambridge, MA, USA), 500 ng E2 (UbcH5c, Boston Biochem, E2-627), 10 μg Ubiquitin (Sigma-Aldrich, U6235), 2mM ATP (Cell signaling, 9804) in the absence and presence of FLAG-TRIM25 wild type or C.S. mutant in 60 μl of reaction buffer (40mM Tris-HCl, pH 7.6, 50mM NaCl, and 1mM DTT) for 1 h at 37° C. After incubation, reactions were pulled-down by centrifugation and washed four times with binding buffer, followed by western blotting using a-Ub-HRP or a-PPARγ mouse antibodies. Reactions were directly subjected to western blotting using a-TRIM25 antibodies to see TRIM25 self-ubiquitination as a control.

Animals

All animal experiments were performed according to procedures approved by Ulsan National Institute of Science and Technology's Institutional Animal Care and Use Committee. Five-week-old male C57BL/6 J mice (DBL, Korea) were fed a high-fat diet (60% kcal fat, D12492, Research Diets Inc., NJ, USA) for 10 weeks.

2-3. Results

TRIM25 directly interacts with PPARγ

To identify potential PTM modulators of PPARγ, we performed proteomic analyses of binding complexes formed with PPARγ. As shown in Fig. 1A, Among multiple PPARγ-associated proteins, TRIM25 was of particular interest because it functions as an ubiquitin E3 ligase and as an ISG15 E3 ligase.[48] It has been also reported that TRIM25 involves in innate immune defense against viruses by mediating ubiquitination of DDX58. It mediates Lys-63-linked polyubiquitination of the DDX58 N-terminal CARD-like region which is crucial for triggering the cytosolic signal transduction that leads to the production of interferons in response to viral infection.[20] These data led us to investigate whether TRIM25 has physiological roles in regulating both PTM and transcriptional activity of PPARγ. To confirm the interaction between TRIM25 and PPARγ, PPARγ were immunoprecipitated from cultured adipocytes and detected TRIM25 by immunoblotting. As shown in Fig. 1B, PPARγ interacts with TRIM25. We further investigated the interaction between TRIM25 and PPARγ *in vitro*, using recombinant PPARγ fragments, including the A/B region which is transcriptional regulatory region (A/B), DNA Binding Domain region/Hinge region (DBD/H) and Ligand Binding Domain (LBD). As shown in Fig. 1C, TRIM25 directly interacted with the DBD/H domain of PPARγ. To further determine the specific region required for TRIM25 binding to PPARγ, recombinant glutathione S-transferase (GST)-fused dissected smaller region of TRIM25 fragments were generated. Coimmunoprecipitation showed that amino acids 350-400 region of TRIM25 ($TRIM25^{350\text{-}400}$) is necessary for its interaction with PPARγ (Fig. 1D).

The E3 ligase activity of TRIM25 is required to decrease PPARγ protein stability

It has been reported that TRIM25 is known as an E3 ligase, we next examined whether TRIM25 regulates protein stability of PPARγ. To further elucidate the role of TRIM25 in PPARγ stability as an E3 ligase, we generated E3 ligase-defective TRIM25 mutant by changing cysteine 50 residue to serine (C50S) within TRIM25 ($TRIM25^{CS}$) which is crucial for E3 ligase.[32] When $TRIM25^{CS}$ mutant was overexpressed with PPARγ, it still interacted with PPARγ but did not induce PPARγ degradation, (Fig. 2A). Furthermore, with an increase of $TRIM25^{WT}$ expression does dependently decreased PPARγ protein level, but $TRIM25^{CS}$ mutant did not affect (Fig. 2B). These results indicated that E3 ligase activities of TRIM25 are involved in PPARγ degradation. Moreover, we also examined which region is crucial to TRIM25-mediated PPARγ degradation. As shown in Fig

1D, full length of TRIM25 (TRIM25$^{1-630}$) and TRIM25$^{1-449}$ fragment containing PPARγ binding region as well as E3 ligase activity produced PPARγ degradation, whereas other fragments including TRIM25$^{1-185}$, TRIM25$^{400-630}$, TRIM25$^{350-630}$, and TRIM25$^{81-630}$ without PPARγ binding region or E3 ligase catalytic activity failed to induce PPARγ degradation (Fig. 2C).These results indicate that TRIM25 mediated PPARγ degradation is required for E3 ligase activity of TRIM25 as well as interaction between TRIM25 and PPARγ. Interestingly, forced expression of TRIM25 significantly reduced the protein level of PPARγ, which was recovered and interaction between PPARγ and TRIM25 was also increased by treatment with MG132, a specific proteasome inhibitor (Fig. 2D). Additionally, treatment with MG132 suppressed TRIM25-mediated degradation of PPARγ (Fig. 2E). Next, we analyzed the kinetics of the protein levels of PPARγ regulated by TRIM25 using cyclohexamide (CHX), an inhibitor of protein translation. TRIM25^{WT} was shown to reduce the half-life of PPARγ compared to TRIM25^{CS} mutant in 3T3-L1 adipocytes (Fig. 2F), suggesting that TRIM25 decreases the half-life of PPARγ expression via proteasomal degradation.

TRIM25 mediates ubiquitination of PPARγ as an E3 ligase

We performed ubiquitination analysis to further test whether TRIM25 functions as a specific E3 ligase of PPARγ. Retrovirus expressing TRIM25^{WT} or TRIM25^{CS} mutant was transduced into fully differentiated adipocytes, then we immunoprecipitated endogenous PPARγ cultured adipocytes and detected ubiquitination of endogenous PPARγ by western blotting (Fig. 3A). Although the protein levels of PPARγ was decreased by TRIM25^{WT}, TRIM25^{WT} resulted in increased PPARγ ubiquitination under pretreatment with MG132. However, TRIM25^{CS} was not able to induce PPARγ ubiquitination. Consistent with these results, transiently expressed TRIM25^{WT} more enhanced PPARγ ubiquitination compared to TRIM25^{CS} mutant in HEK-293 cells (Fig. 3B). To further confirm the effect of TRIM25 on PPARγ , we next performed *in vitro* ubiquitination assay using purified TRIM25 and PPARγ. As expected, TRIM25 also induce ubiquitination of recombinant PPARγ (Fig. 3C), whileTRIM25^{CS} did not (Fig. 3D). These results suggest that PPARγ is a substrate of TRIM25.

Expression of TRIM25 is decreased during adipogenesis

In present study, our results demonstrated that TRIM25 regulated PPARγ protein stability. Thus, we next further investigate the physiological role of TRIM25 in adipocyte differentiation. At

first, we examined the physiological relevance of TRIM25 and PPARγ during adipocyte differentiation. Western blot analysis and real-time PCR analysis revealed that TRIM25 was expressed in pre-adipocytes and its expression was significantly decreased during adipogenesis (Fig. 4A and B). Contrast with TRIM25 expression, PPARγ and adipogenic marker genes, including aP2 and adiponectin were significantly increased, indicating that TRIM25 expression is negatively correlated with that of PPARγ. Furthermore, we also measured TRIM25 expression in adipose tissue from diet-induced obesity model mouse (DIO mouse) and genetically induced obesity model mouse (*ob/ob* mouse. Fig. 4C and D showed that TRIM25 expression significantly down regulated, while PPARγ expression was dramatically up-regulated in adipose tissue from both DIO mouse and *ob/ob* mouse compared with control mouse. Together, these results suggest that the expression of TRIM25 inversely correlated with that of PPARγ *in vitro* and *in vivo*.

TRIM25 suppresses adipocyte differentiation

PPARγ is both necessary and sufficient for adipogenesis, and alterations in PPARγ activity affect adipogenesis.[30] Consistent with TRIM25-mediated regulation of PPARγ stability, differentiation of 3T3-L1 cells was suppressed in expressed $TRIM25^{WT}$, mature adipocytes was evaluated by determination of triglyceride (TG) accumulation using Oil Red O staining (Fig. 5A). Furthermore, the protein and mRNA expression of adipocyte-selective genes, including aP2, CCAAT/enhancer-binding protein a (C/EBP a), adiponectin, adipsin, lipoprotein lipase (LPL), and glucose transporter-4 (GLUT4), were also reduced (Fig. 5B and 5C). However, $TRIM25^{CS}$ mutant did not alter expression of adipogenic markers. In addition, specific knockdown of TRIM25 enhanced adipocyte differentiation (Fig. 6A) and the expression of adipocyte-specific proteins and genes compared with specific knockdown of TRIM25 using a lentivirus expressing a short hairpin (sh) RNA targeting TRIM25 (Fig. 6B and 6C). To investigate the effect of the TRIM25 on PPARγ transcriptional activity, we performed dual luciferase system in HEK-293 cells expressing a PPRE-containing luciferase construct. As shown in Fig. 5D, overexpression of $TRIM25^{WT}$ suppressed PPARγ transcriptional activity of PPARγ, but $TRIM25^{CS}$ did not affect. Consistent with this result, specific knockdown of TRIM25 promoted PPARγ transcriptional activity (Fig. 6D). To further confirm the effect of TRIM25 in adipogenesis, we used wild type and TRIM25 knock-out (KO) mouse embryonic fibroblasts (MEFs). Compared to WT MEFs, KO MEFs exhibited dramatically enhanced lipid accumulation (Fig. 7A). Furthermore, the expression of adipogenic markers significantly increased in KO MEFs compared to WT MEFs (Fig. 7B and 7C). Taken together, these results suggest that TRIM25 plays a key role in the regulation of PPARγ-dependent adipogenic processes.

2-4. Discussions

Adipose tissue is the center of regulating systemic metabolism in rodent and human. Dysregulation of adipocyte differentiation and/or physiological functions result in metabolic disorders such as type 2 diabetes.[25] As PPARγ functions as a master regulator of fat cell differentiation and glucose/lipid metabolism, understanding mechanisms of PPARγ regulation is crucial for combating metabolic disorders. PPARγ activity is regulated by its ligand as well as by modulating its protein level. PPARγ (+/−) heterozygous mice are protected from high-fat diet-induced weight gain and insulin resistance.[49-51] These findings suggest that abundance of PPARγ proteins itself in adipose tissue can also ameliorate obesity and insulin resistance.

The ubiquitin-proteasome pathway involves an enzymatic cascade, through which multiple ubiquitin molecules are covalently attached to a substrate. The polyubiquitinated substrate is then recognized by the 26S proteasome and degraded. Ubiquitin is shuttled to the substrate via a series of enzymatic transfers originating with the ubiquitin activating enzyme (E1) and ending with modification of the substrate by the ubiquitin polypeptides, catalyzed by one or more ubiquitin ligases (E3).[52] The E3 ubiquitin ligases determine the specificity of substrate selection by the ubiquitin-proteasome system.[20, 48] PPARγ protein stability and activity are also controlled by the ubiquitin proteasome system. Thus, identification of the ligase or ligases required for ubiquitination of PPARγ in adipocyte is essential for understanding the physiological significance of PPARγ ubiquitin modification in adipocytes. In this study, we showed TRIM25 is direct E3 ligase to target PPARγ. TRIM25 reduced PPARγ protein levels through direct interaction through DBD/H domain within PPARγ and TRIM25$^{350\text{-}400}$ region. In addition to direct interaction, E3 ligase catalytic activity is also essential for TRIM25-mediated regulation of PPARγ stability and adipocyte differentiation. Thus, our results demonstrate that TRIM25 is a novel regulator of PPARγ, mediating ubiquitination and degradation of PPARγ. Interestingly, poly-ubiquitination process of PPARγ is not always lead to proteasomal degradation and down regulation of PPARγ activity.[33-35] Of course, in our study, TRIM25 induced ubiquitination and degradation of PPARγ and suppressed adipocyte differentiation. Furthermore, MKRN1 mediated PPARγ ubiquitination and degradation to target for ubiquitination at lysine 184 and 185 residues in PPARγ.[53] FBXO9 directly interacted with PPARγ through the activation function-1 domain and ligand-binding domain, which also decreased the protein stability of PPARγ through induction of ubiquitination.[54] In contrast, NEDD4 interacts with the hinge and LBD of PPARγ and increases ubiquitination but it also increases PPARγ stability to inhibit proteasomal degradation.[55] Siah2 causes PPARγ degradation but it also up-regulates ligand-dependent activation of PPARγ.[56] tripartite motif protein 23 (TRIM23) regulates PPARγ ubiquitination to stabilize it.[57] These

findings demonstrate important roles for E3 ligases in PPARγ posttranslational regulation, but it is not unclear to make different results in PPARγ stability. Therefore, further studies are required to understand how ubiquitination affect PPARγ protein stability and activity. We will examine how TRIM25 plays a central role on PPAR*γ* in a ligand-dependent manner for future studies.

In this study, we found TRIM25 as binding protein of PPARγ in adipocyte and their expression showed inverse correlation in adipose tissue and adipocyte (Fig 4). Interestingly, TRIM25 and PPARγ are not only expressed in adipocytes but also in other tissue. And, interestingly, they also have inversed function in cancer. Many studies have been reported PPARγ as a tumor suppressor because it has a role of inflammation and glucose metabolism in cancer.[58-61] Overexpression of PPARγ suppressed survival to inhibit cell proliferation and tumor growth.[62-63] In contrast, silence of PPARγ in cancer has reverse effects to activate cancer survival pathway. [64-67] TRIM25 also has been studying related several cancers. Especially, TRIM25 is over-expressed in breast, ovarian and lung cancer, while PPARγ is down regulated in these cancer compare with non-cancer line.[68-69] These results shows that the possibility that TRIM25 also related in estrogen target cancer by regulating PPARγ stability as tumor suppressor. After study that targets TRIM25 may advance in the development of novel therapy for selective PPARγ stability and activity as modulators.

Figures

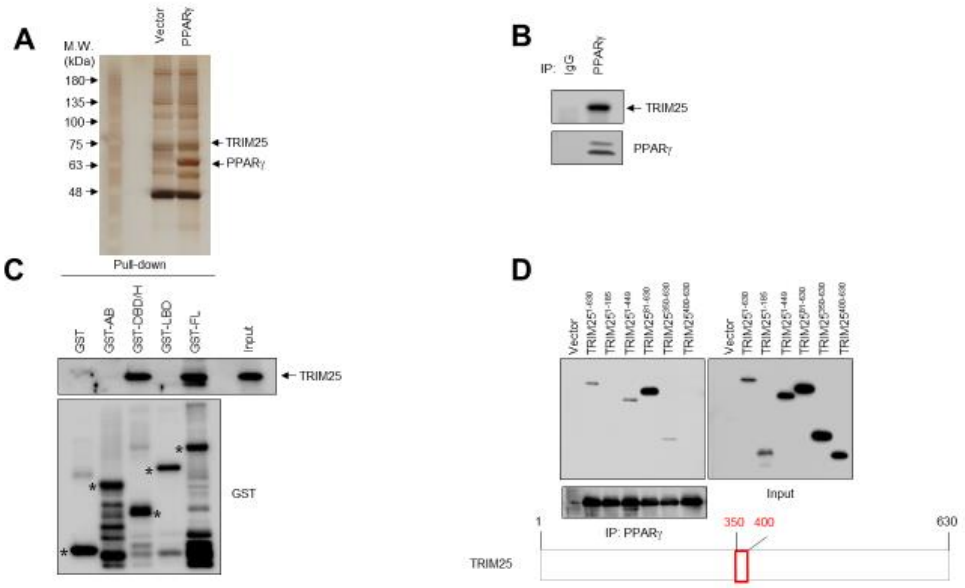

Figure 1. TRIM25 directly interacts with PPARγ

(A) The silver staining of PPARγ and its associated proteins. Flag-tagged PPARγ and empty vector were expressed PPARγ-deficient mouse embryonic fibroblasts (MEFs). At day 6 of differentiation, cells were homogenized, and the nuclear extracts were used for immunoprecipitation. PPARγ complexes were visualized by silver staining and analyzed by LC MS/MS.

(B) Endogenous PPARγ was immunoprecipitated with anti- PPARγ antibody. The precipitates were analyzed with anti-TRIM25 antibody.

(C) GST-fused A/B, LBD, DBD/H, and full-length PPARγ proteins were incubated with TRIM25-expressing cell lysates. The complexes of GST-fragments were isolated using GSH Sepharose, and analyzed by western blotting with anti-TRIM25 antibody.

(D) GST-fused TRIM25 fragments ($TRIM25^{1-630}$, $TRIM25^{1-185}$, $TRIM25^{1-449}$, $TRIM25^{81-630}$, $TRIM25^{350-630}$, and $TRIM25450^{-630}$) were incubated with recombinant PPARγ protein. The TRIM25 complexes were isolated using GSH Sepharose, and analyzed by western blotting using anti-PPARγ and TRIM25 antibodies.

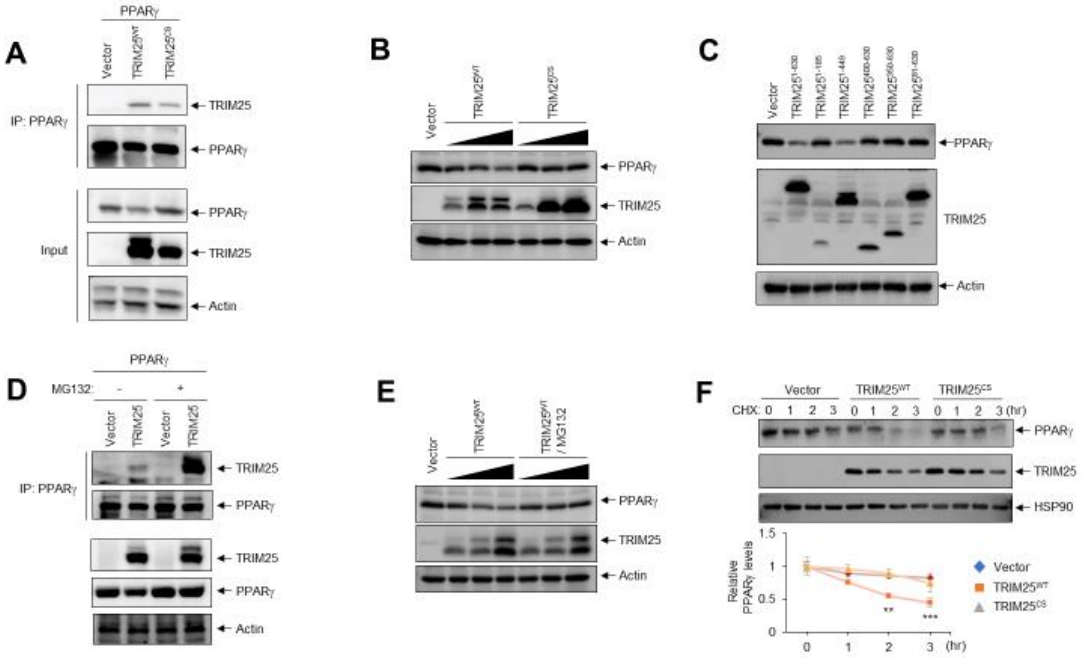

Figure 2. E3 ligase activity of TRIM25 is necessary for its ability for regulating PPARγ stability

(A) Interaction between exogenous TRIM25^{WT}, TRIM25^{CS} and PPARγ. HEK-293 cells were transfected with the plasmids expressing FLAG-PPARγ and HA- TRIM25^{WT}, TRIM25^{CS} plasmids. The transfected cells were then harvested and WCE were immunoprecipitated using FLAG antibodies, followed by western blotting using FLAG and HA antibodies.

(B) GST-fused TRIM25 fragments (TRIM25$^{1-630}$, TRIM25$^{1-185}$, TRIM25$^{1-449}$, TRIM25$^{81-630}$, TRIM25$^{350-630}$, and TRIM25450^{-630}) were incubated with recombinant PPARγ protein. The TRIM25 complexes were isolated using GSH Sepharose, and analyzed by western blotting using anti-PPARγ and TRIM25 antibodies.

(C) HEK-293 cells, transfected with the expressing PPARγ (2 μg), TRIM25^{WT}, TRIM25^{CS} (0.5 μg) and increasing concentrations of HA- TRIM25^{WT}, TRIM25^{CS} (0.5 and 1 μg), were treated. The proteins were detected by western blotting.

(D) Interaction between exogenous TRIM25 and PPARγ. HEK-293 cells were transfected with the plasmids expressing FLAG-PPARγ and HA-TRIM25 plasmids with or without 10 μM MG132 for 6 h. The transfected cells were then harvested and WCE were immunoprecipitated using FLAG antibodies, followed by western blotting using FLAG and HA antibodies.

(E) Effects of MG132 on TRIM25-mediated PPARγ degradation. HEK-293 cells, transfected with the expressing PPARγ (2 μg), TRIM25 (0.5 μg) and increasing concentrations of HA-TRIM25 (0.5 and 1 μg), were treated with 10 μM MG132 for 6 h. The proteins were detected by western blotting.

(F) Half-life of PPARγ in the presence of TRIM25. HEK-293 cells were transfected with plasmids expressing indicated proteins followed by treatment with CHX. WCE were perceived by western blotting.

All represented error bars are S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

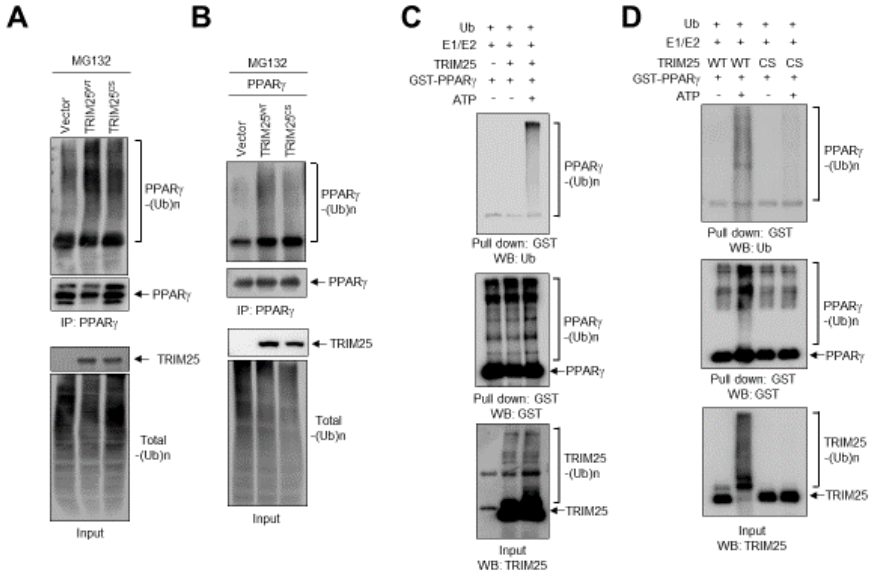

Figure 3. E3 ligase of TRIM25 is required for PPARγ ubiquitination

(A) PPARγ ubiquitination by active TRIM25 E3 ligase. 3T3-L1 cells, transfected FLAG-PPARγ and HA- TRIM25^{WT}, TRIM25^{CS} plasmids with 10 μM MG132 for 6 h. The transfected cells were then harvested and WCE were immunoprecipitated using FLAG antibodies, followed by western blotting using Ubiquitin and FLAG, HA antibodies.

(B) HEK-293 cells, transfected HA- TRIM25^{WT}, TRIM25^{CS} plasmids with 10 μM MG132 for 6 h. The transfected cells were then harvested and WCE were immunoprecipitated using PPARγ antibodies, followed by western blotting using Ubiquitin and FLAG, HA antibodies.

(C-D) Ubiquitination of PPARγ by TRIM25 in vitro. Purified recombinant GST- PPARγ was incubated with E1, E2, ubiquitin (Ub) and ATP in the absence and presence of HA- TRIM25^{WT} or/and TRIM25^{CS} as indicated followed. Reactions were boiled in 1% SDS to disrupt protein interaction, followed by immunoprecipitation using PPARγ antibodies. Ubiquitination of PPARγ was analyzed by western blotting using Ub or PPARγ antibodies. For input, ubiquitin reactions were directly subjected to western blotting using TRIM25 antibodies.

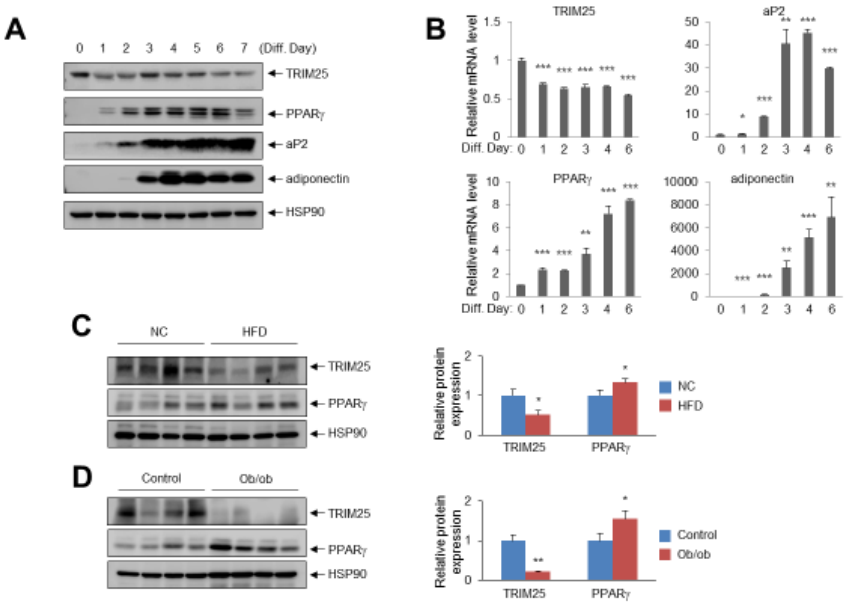

Figure 4. Reverse correlation between TRIM25 and PPARγ expression

(A-B) 3T3-L1 pre-adipocytes were induced to undergo adipogenesis and the cells were harvested each day as indicated. (A), WCE were harvested and followed by western blotting as indicated antibodies. (B), RNA isolated and subjected to real-time PCR to determine the level of TRIM25 and indicated mRNA expressions.

(C-D) TRIM25 and PPARγ expressions in WAT. Quantification of PPARγ compared to TRIM25 was performed to use WAT of High fat diet mouse and Ob/Ob mouse. (n=4). (C), Lysates of tissues were followed by western blotting using indicated antibodies. (D), RNA isolated and subjected to real-time PCR to determine the level of TRIM25 and PPARγ mRNA.

All represented error bars are S.E.M. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with control.

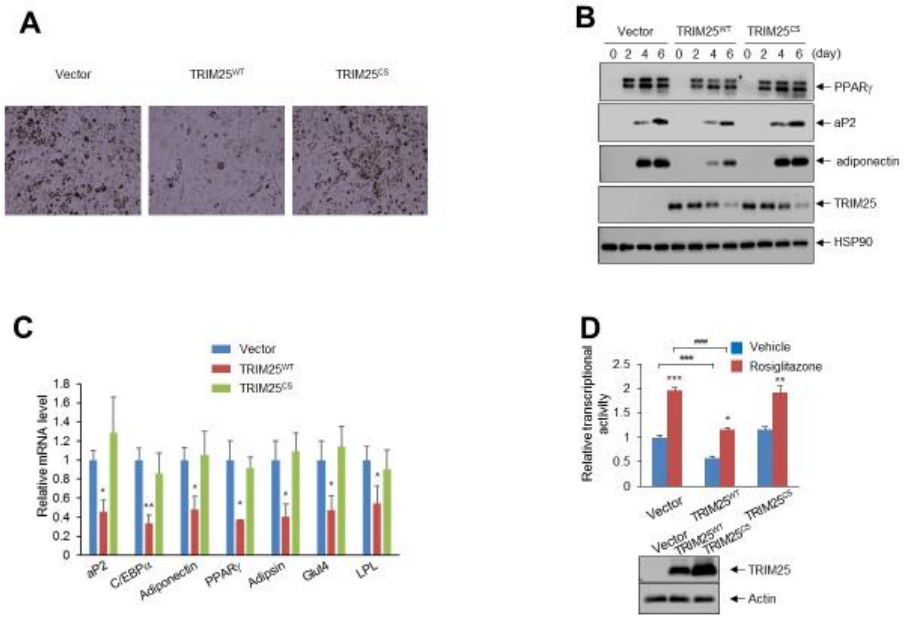

Figure 5. TRIM25 suppresses adipocyte differentiation in 3T3-L1

(A-C) Empty vector, TRIM25^{WT} and TRIM25^{CS} expressing 3T3-L1 cells were induced adipocyte differentiation for 6 days. (A), Cells were stained with Oil Red O. (B), These cells were then harvested and WCE were followed by western blotting as indicated above and (C), analyzed mRNA expression of adipogenic genes.

(D) Transcriptional activity of a PPAR-derived reporter gene in HEK-293 cells following overexpression TRIM25^{WT} and TRIM25^{CS}. The cells were incubated with or without rosiglitazone (10μM) for 24 hr. harvested, and quantified firefly luciferase. WCE were perceived by western blotting and their levels measured.

All represented error bars are S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

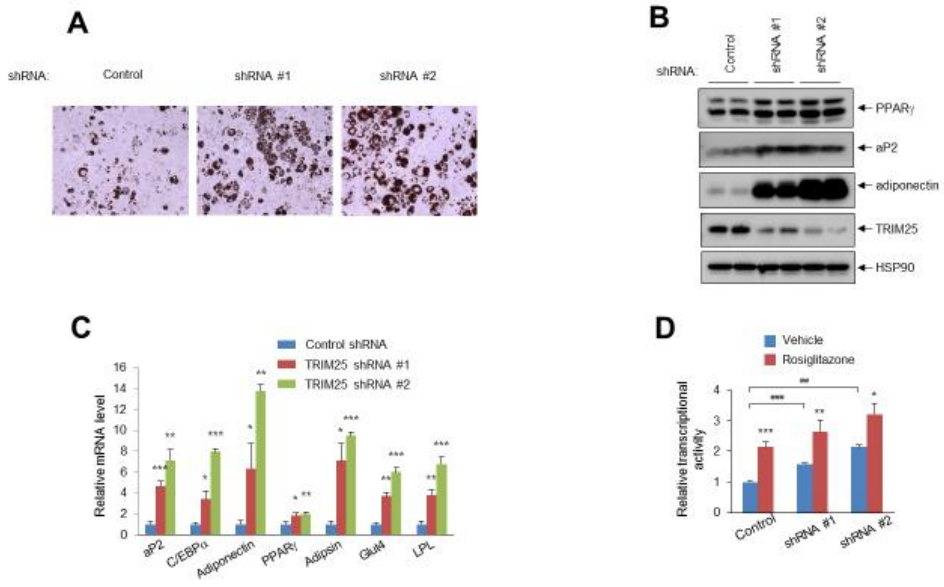

Figure 6. Knockdown of TRIM25 increases adipocyte differentiation in 3T3-L1

(A-C) Scr shRNA or shRNA against TRIM25 was infected into 3T3-L1 cells, and cells were induced adipocyte differentiation. (A), Cells were stained with Oil Red O. (B), These cells were then harvested and WCE were followed by western blotting as indicated above and (C), analyzed mRNA expression of adipogenic genes.

(D) Transcriptional activity of a PPAR-derived reporter gene in HEK-293 cells following Scr shRNA or shRNA against TRIM25 was infected. The cells were incubated with or without rosiglitazone (10μM) for 24 hr. harvested, and quantified firefly luciferase.

All represented error bars are S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

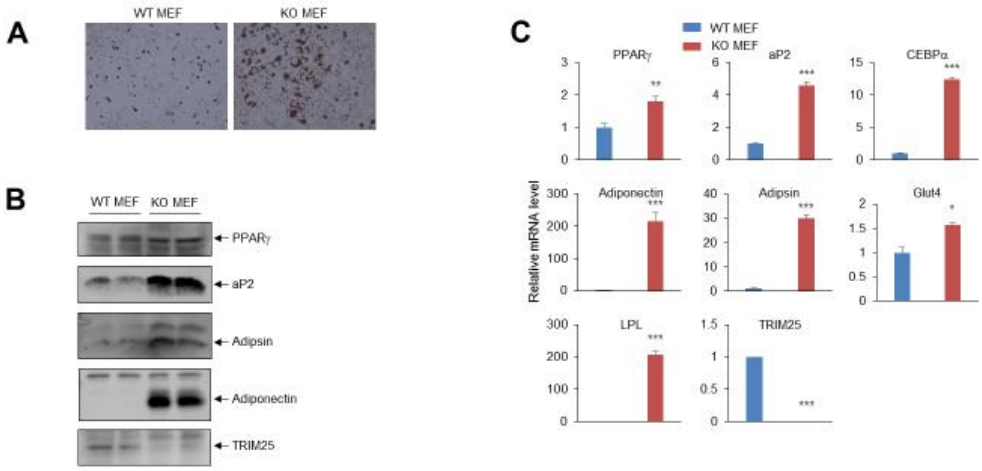

Figure 7. TRIM25 regulates adipocyte differentiation in MEFs

(A-C) Mouse embryonic fibroblast cells (MEFs) and TRIM25 deletion MEFs were induced adipocyte differentiation. (A), Cells were stained with Oil Red O. (B), These cells were then harvested and WCE were followed by western blotting as indicated above and (C), analyzed mRNA expression of adipogenic genes.

All represented error bars are S.E.M. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control.

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